REMARKS

Interview

Applicants would like to thank Examiner Nora Rooney for the interview held with Applicants' representative on July 22, 2011. During the interview, the enablement and written description rejections and proposed amendments to claim 1 were discussed. The Examiner stated that the proposed amendments to claim 1 would overcome the rejections under 35 U.S.C. § 112, first paragraph (enablement and written description) of claim 1. However, the Examiner also stated that the proposed amendments to claim 1 would require a further search and examination for relevant prior art. Accordingly, Applicants are filing a Request for Continued Examination with this Amendment and incorporating the proposed amendments to claim 1 discussed during the interview into the new claims.

Status of the Claims

Claims 1-19 have been canceled without prejudice or disclaimer of the subject matter claimed therein. New claims 20-37 replace claims 1-19. Support for new claims 20-37 can be found throughout the specification. Representative support is summarized in the table below.

Claim(s)	Representative Support
20	Paragraph 0029 and Examples
21, 22	Claim 1 and Paragraph 0016
23-26	Paragraph 0017
27-29	Paragraphs 0018 and 0019
30	Claim 9
31	Claim 10
32	Claim 11
33	Claim 12

34	Claim 13
35	Claim 14
36	Claim 18
37	Claim 19

New claims 20-37 do not introduce prohibited new matter.

Applicants submit that new claims 20-35 are directed to the same invention as claims 1-17, and therefore, are currently under examination.

New claims 36 and 37 are withdrawn from consideration as being directed to a separate invention. Claims 36 and 37 are directed to methods of making and using the microspheres of claim 21. Applicants respectfully point out that MPEP 821.04(b) requires that once a product claim is found allowable, withdrawn method claims which depend from or otherwise include all the limitations of the allowable product claim be rejoined. Thus, once a claim directed to a product (claims 20-35) is found allowable, withdrawn method claims (claims 35 and 36) which depend from or otherwise include all the limitations of the allowed claim must be rejoined.

Rejection Under 35 U.S.C. § 112, First Paragraph

A. Claims 1, 2, 4, and 9-17 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly being enabling only for PLGA microspheres containing birch pollen extract wherein the microspheres have lectins on their surface.

Without acquiescing to the propriety of the rejection, claims 1, 2, 4, and 9-17 have been canceled and replaced with new claims 20-35 in the interest of advancing prosecution of the application. This rejection is not applicable to new claims 20-35 because the specification teaches how to make and use the claimed invention recited in the new claims.

Moreover, during the interview, the Examiner stated that she would withdraw this rejection with respect to claim 1, if claim 1 were amended to delete the recitation of "antigen and/or DNA of antigens" and "binding constant." The proposed amendments have been incorporated into new claim 20. Accordingly, the present rejection is not applicable to new claims 20-35.

Applicants submit that the specification teaches how to make and use microspheres

comprising Aleuria aurantia lectin (AAL) on their surface and how to make and use such microspheres comprising an allergen. The specification describes in detail and provides sufficient guidance as to how to make and use such microspheres. As an example, in paragraphs 0034-0045, the specification provides adequate guidance for making the microspheres containing AAL on their surface and for making such microspheres comprising an allergen. For example, in paragraphs 0010-0013, the specification teaches the use of the microspheres comprising allergens and AAL on their surface to treat allergies. Moreover, as described in detail in Examples 3 and 4, microspheres containing an allergen and AAL on their surface selectively target and bind human epithelial cells. Moreover, Example 1 shows that an allergen administered orally to animal using microspheres containing AAL on their surface effectively induces an immune response in animals. Further, Example 2 shows that the antigenicity of an allergen packed into microspheres containing AAL on their surface is not lost after gastric digestion. Therefore, the specification provides adequate guidance for making and using microspheres containing AAL on their surface and such microspheres comprising an allergen.

The Office Action alleges that Brayden teaches that human M-cells are unlikely to show specific UEA-1 lectin binding. Applicants respectfully point out that Brayden's statements are directed to UEA-1 lectin. UEA-1 is a different lectin than AAL. It is known that UEA-1 lectin is toxic and cannot be used in humans. Unlike UEA-1, AAL is not toxic. Moreover, Brayden does not state that microspheres containing AAL on their surface will not be effective as a delivery vehicle of allergens. Brayden only states that ". . . it will be interesting to see if regulatory authorities will permit human Phase I testing of a UEA-1 mimetic with an antigen loaded particle, even if murine immune data has turned out to be positive." Brayden may have expressed doubt as to whether the regulatory authorities will permit human Phase I testing of a UEA-1 mimetic because UEA-1 is toxic. Brayden's statement does not imply that microspheres containing AAL, which is not toxic, cannot be used to deliver allergens to subjects. In fact, Brayden confirms that AAL is effective as a vehicle for delivering allergens to animals.

The Office Action also alleges that it is not clear whether UEA-1 can effectively target human M-cells based on the teachings of Azizi. As mentioned above, UEA-1 is structurally different from AAL. UEA-1 and AAL are also functionally different because UEA-1 and AAL bind different subtypes of fucose (see attached abstract from Vector Labs catalogue). Moreover, Applicants respectfully point out that like Brayden, Azizi does not

state that microspheres containing AAL cannot be used as a vehicle for delivering allergens. Azizi only states that the receptors for human M-cells have not been discovered.

Applicants respectfully submit that the inventors of the present invention have shown that microspheres comprising AAL on their surface are effective vehicles for delivering allergens to mammals. The inventors have also shown that AAL binds human epithelial cells. As an example, microspheres comprising AAL bind human intestinal epithelial cells from Caco-2 (see Examples 3 and 4). Caco-2 cells are derived from human colorectal carcinoma and have surface properties that are representative of human intestinal epithelium (see paragraph 0023 of specification). Applicants respectfully submit that Azizi pointed out that Caco-2 cells have been used for studying "the morphology and expression of M cell surface markers and antigen absorption, and to screen oral drug/vaccine delivery systems as it closely imitates human cells" (see Azizi page 4, right column). Accordingly, Caco-2 cells are a well-known model for studying vaccine delivery. It is acknowledged that Azizi believes that this model may be an "over-simplification" of *in vivo* events, but Caco-2 cells are at present being used as a model for human M-cells for drug/vaccine delivery system.

Moreover, the reference of Roth-Walter (Roth Walter *et al.* J. Allergy Clin. Immunol., 2004, 114 (6), 1362-1368, attached) confirms that microspheres comprising AAL on their surface are effective in targeting allergen to M-cells by showing that orally administered microspheres comprising birch pollen and AAL on their surface induced an immune response in mice. The reference of Roth-Walter also confirms that AAL binds Caco cells (see abstract and page 1365, left column).

Further, at the time of the invention, it was thought that the lectin binding pattern of M-cells and Caco-2 cells is the same. Brinck (Brinck *et al.* Histol. Histopathol. 1995, 10, 61-70, attached) reported that they found "no differences between the lectin-binding pattern of M-cells and enterocytes in the follicle-associated epithelium." Enterocytes are the intestinal epithelial cells. Accordingly, there is no evidence that suggests that microspheres containing AAL will not bind epithelial cells and will not act as an effective vehicle for delivering allergens.

Applicants respectfully submit that the present claims are directed to microspheres comprising AAL on their surface, which are product or composition claims. It is also pointed out that the claim directed to a method of treating an allergy in a subject, at this time, is withdrawn from consideration as being directed to a separate invention.

The specification teaches how to make and use microspheres comprising AAL on

their surface and how to make and use such microspheres comprising allergens. The specification describes in detail how to make microspheres comprising AAL on their surface (see for example paragraphs 0034-0041) and provides Examples showing the effective oral delivery of allergens using microspheres containing AAL on their surface and the induction of an immune response in mice. Accordingly, the specification enables the claimed invention.

B. Claims 1, 2, 4, and 9-17 are rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not adequately described in the specification.

Without acquiescing to the propriety of the rejection, claims 1, 2, 4, and 9-17 have been canceled and replaced with new claims 20-35 in the interest of advancing prosecution of the application. This rejection is not applicable to new claims 20-35 because the specification adequately describes the invention recited in the new claims.

Applicants respectfully point out that the proposed amendments to claim 1 discussed during the interview have been incorporated into new claim 20. The Examiner indicated during the interview that the rejection of claim 1 would be withdrawn if claim 1 were amended as discussed during the interview. Accordingly, Applicants submits that the present rejection is not applicable to new claims 20-35.

The specification provides adequate written description for new claims 20-35. The specification describes microspheres comprising AAL in detail for example at paragraphs 0024-0029, 0032-45, 0052, and Examples 1-4. The specification also describes the different allergens that could be included in the microspheres in paragraphs 0016-0019 and Examples 1-4. As discussed above, the Examples show that microspheres comprising AAL bind to murine and human epithelial cells.

Conclusion

The foregoing amendments and remarks are being made to place the application in condition for allowance. Applicants respectfully request entry of the amendments, reconsideration, and the timely allowance of the pending claims. A favorable action is awaited. Should an interview be helpful to further prosecution of this application, the Examiner is invited to telephone the undersigned.

If there are any additional fees due in connection with the filing of this response, please charge the fees to our Deposit Account No. 13-3250. If a fee is required for an

extension of time under 37 C.F.R. §1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit Account.

Respectfully submitted,
MILBANK, TWEED, HADLEY & McCLOY, LLP

Date: October 13, 2011

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Unconjugated Aleuria Aurantia Lectin (AAL)

Catalog #	Unit Size	Product Information	MSDS
L-1390	2 mg	Data Sheet	msdsL1390.pdf

Isolated from *Aleuria aurantia* mushrooms

This lectin is a dimer of two identical subunits of about 36,000 daltons each with an isoelectric point of about pH 9. Unlike Ulex europaeus and Lotus tetragonolobus lectins which prefer (α -1,2) linked fucose residues, Aleuria aurantia lectin binds preferentially to fucose linked (α -1,6) to N-acetylglucosamine or to fucose linked (α -1,3) to N-acetyllactosamine related structures.

Inhibiting/Eluting Sugar: 100 mM L-fucose

M cell targeting with *Aleuria aurantia* lectin as a novel approach for oral allergen immunotherapy

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Vienna, Austria

Background: The extent and quality of the immune response to orally applied allergens may critically depend on the precise site of uptake at the intestinal mucosa.
Objective: The aim of this study was to construct allergen vehicles optimized for oral allergen immunotherapy.
Methods: By using a murine model, we examined the immunomodulatory effect of birch pollen proteins entrapped in poly(D,L-lactide-co-glycolide) microspheres, which were specifically targeted to enterocytes or to M cells, in an ongoing T_H2 response. BALB/c mice express different carbohydrates on these 2 cell types. To target the sialylic residues on murine enterocytes, we functionalized microspheres with wheat germ agglutinin (WGA) and, to target α-L-fucose on M cells, with a lectin from *Aleuria aurantia* (AAL), the orange peel mushroom.
Results: Both WGA and AAL functionalization enhanced binding to human Caco2 cells substantially, which express sialylic and, as carcinoma cells, also α-L-fucose residues. Different groups of BALB/c mice were first sensitized to birch pollen and subsequently fed with birch pollen-loaded functionalized (WGA microspheres, AAL microspheres) or nonfunctionalized, birch pollen extract–loaded particles. When mice were fed with AAL microspheres, birch pollen-specific IgG2a, but not IgG1 or IgE, increased significantly. As expected, in a ^{3}H-thymidin assay, their splenocytes proliferated specifically on birch pollen stimulation. Both targeting strategies, using WGA or AAL, induced IL-10 as well as IL-4 production. However, in AAL microsphere–treated mice, IFN-γ synthesis was significantly increased, which may be responsible for the significant IgG2a production in this group.
Conclusion: Our data indicate that targeting M cells by using AAL-coated allergen vehicles may be a promising strategy for oral allergen immunotherapy. (J Allergy Clin Immunol 2004;114:1362-8.)

Key words: Aleuria aurantia *lectin, M cell, gastrointestinal tract, oral immunotherapy, allergy, birch pollen, microspheres, PLGA,* Salmonella typhimurium

Many pathogens like *Salmonella typhimurium* infect via the intestinal route by adhering to M cells, which have a sampling function in the intestine and overlay directly Peyer patches of the organized mucosal-associated lymphoid tissues (MALTs). M cells are a differentiated epithelial cell subset within the specialized epithelium covering the organized lymphoid tissues, termed *follicle-associated epithelium.* Although the membranous epithelial M cells perform predominantly antigen sampling, they are also exploited as a route of host invasion by many pathogens.[1,2] Such known pathogens are *S typhimurium,*[3] *Yersinia enterocolitica,*[4] and reoviruses.[5]

In mice, M cells, but not enterocytes, express α-L-fucose residues on their apical surface. Our working hypothesis was that it should be possible to exploit this route for allergen immunotherapy when targeting the allergens to M cells. We chose *Aleuria aurantia* lectin (AAL) from the edible orange cup mushroom that has α-L-fucose specificity.[6] In addition, the crystal structure[7,8] and physical properties are very similar to the neuramidase of *S typhimurium.*[6,9] Because *Salmonella* specifically binds to M cells, these species are already used as antigen vehicles for several proteins and DNA vaccines.[10] However, although the applied strains are nonpathogenic, mild diarrhea is observed regularly in the patients.[11]

To avoid the usage of a potentially harmful carrier for oral allergen immunotherapy, we decided to construct a synthetic vehicle for oral allergen immunotherapy with the following properties: (1) adjuvant and carrier function for oral transport of allergen, (2) specific targeting properties to M cells, and (3) from nontoxic origin. As vehicle for allergen immunotherapy, poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles were already successfully

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Abbreviations used
AAL: *Aleuria aurantia* lectin
BP: Birch pollen extract
FITC: Fluorescein isothiocyanate
MALT: Mucosal-associated lymphoid tissue
PLGA: Poly(D,L-lactic-co-glycolic acid)
WGA: Wheat germ agglutinin

used in the subcutaneous route.[12] PLGA is a synthetic copolyester of glycolic and lactic acid with excellent biocompability.[13] Its degradation products are constituents of the natural metabolism and, therefore, biocompatible. For these reasons, PLGA is also used as surgical suture.

Here, we generated PLGA microspheres filled with birch pollen extract (BP) and functionalized them with either AAL or wheat germ agglutinin (WGA), as control lectin that binds to sialylic residues express on normal enterocytes.[14-17] In a BALB/c mouse model, in which M cells express α-L-fucose, we investigated the humoral and cellular immune response after oral feedings with allergen microspheres.

METHODS

Animals

Female BALB/c mice 6 to 8 weeks old were obtained from the Institute for Laboratory Animal Science and Genetics, University of Vienna. All experiments were performed according to European Community rules of animal care and with permission (GZ 66.009/211-Pr/4/2003) of the Austrian Ministry of Science.

Preparation of birch pollen–loaded microspheres

Microspheres of the PLGA H-type (Boehringer Ingelheim, Ingelheim, Germany) were prepared as described.[18] Proteins of birch pollen (Sigma, St Louis, Mo) were extracted and analyzed by SDS-PAGE.[19] Briefly, a water-in-oil (0.15:1) emulsion of 10 mg/mL birch pollen proteins and PLGA was prepared by magnetic stirring and spray-dried. The mean diameter and particle size distribution was examined by laser diffraction and electron microscopy. To determine protein loading, microspheres were dissolved in 50 mmol/L sodium hydroxide containing 1% SDS, and a bicinchonic acid protein assay was performed.

Fluorescein-cadaverine labeling of PLGA

For immunofluorescence microscopy, PLGA was first labeled with 5-([5-aminopentyl] thioureidyl) fluorescein dihydrobromide salt (fluorescein cadaverine) before spray-drying under light protection to prevent bleaching.[20] After activating PLGA in methylenchloride with 33-mmol/L N-hydroxysuccinimide and 23-mmol/L dicyclohexylcarbodiimide, fluorescein-cadaverine in methylenchloride (2 mg/mL) was added and incubated overnight. Excess of unbound fluorescein-cadaverine was then extracted with ice-cold 5-mmol/L hydrochloric acid, followed by precipitation of fluorescein-cadaverine–labeled PLGA with ice-cold methanol. Samples were stored after centrifugation (325*g*, 15 minutes, 10°C) and freeze-dried. For microsphere preparation by spray-drying, fluorescence-labeled PLGA was mixed with unlabeled polymer (wt/wt 1:9).

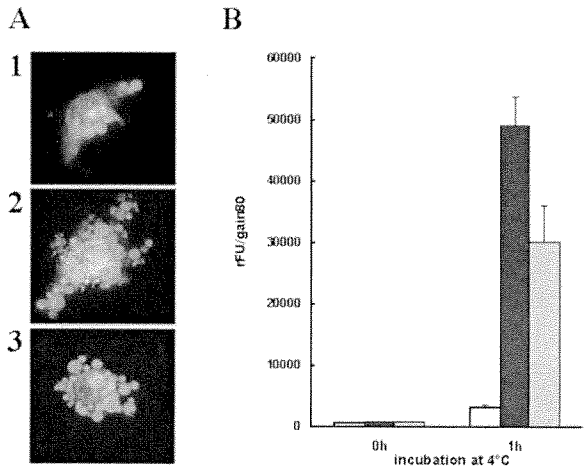

FIG 1. A, Adhesion of bare microspheres *(1),* AAL microspheres *(2),* or WGA microspheres *(3)* to Caco2 intestinal epithelial cells. Caco2 cells were incubated with the different types of fluorescein-cadaverine–labeled microspheres. Plasma membranes were localized with rabbit antialkaline phosphatase antibody and Alexa568 conjugated antirabbit IgG. Nuclei were stained with Hoechst dye. **B,** AAL microparticles *(black bars)* and WGA *(gray bars)* bound substantially better to a Caco2 monolayer than bare microspheres *(white bars).* The intestinal epithelial cell line Caco2 was grown in monolayers and incubated at 4°C with different microsphere preparations containing FITC-cadaverin. Cells with bound microspheres were dissolved and mean fluorescence (n = 3) detected at 485/535 nm. *rFU,* Relative fluorescence units.

Covalent coupling of lectins to the microspheres

For covalent coupling of proteins, the surface carboxylic groups of the polymer were activated by carbodiimide/N-hydroxysuccinimide at a molar ratio of 0.7:1[21] and subsequently incubated with 400 μg/mL AAL, WGA (Vector Laboratories, Burlingame, Calif), or glycine. Excess coupling sites were blocked by adding 10% glycine for 15 minutes.

Tissue cultures and binding studies

Colon carcinoma cells express constitutively α-L-fucose[22] and sialic acid.[15] Therefore, the Caco2 cell line (American Type Culture Collection, Rockville, Md) was chosen for binding studies, and cells were grown in RPMI 1640 cell culture medium containing 10% FCS, 4 mmol/L L-glutamine, and 150 μg/mL gentamycine.

Cells were seeded on tissue culture–treated 96-well microplates at a density of 1×10^5 cells/well. Confluent Caco2 monolayers were washed with ice-cold PBS, pH 7.4, and incubated with 100 μL of a 10 mg/mL suspension of fluorescein-labeled microparticles with or without lectin at 4°C. After rigorous washing to remove nonadherent particles, cells were lysed with 0.1 mol/L sodium hydroxide containing 1% SDS. Cell-associated microspheres were determined fluorometrically (n = 3) at 485/535 nm.

For immunofluorescence studies, cells were grown overnight on coverslips (Nalge Nunc, Naperville, Ill). After binding of fluorescein-labeled microparticles and washing, cells were fixed with 4% paraformaldehyde and blocked with 1% BSA/PBS, and the membrane was visualized on incubation of rabbit antialkaline phosphatase followed by Alexa 568-labeled antirabbit. Nuclei were stained with Hoechst Dye/PBS, and cells embedded in mounting Medium.

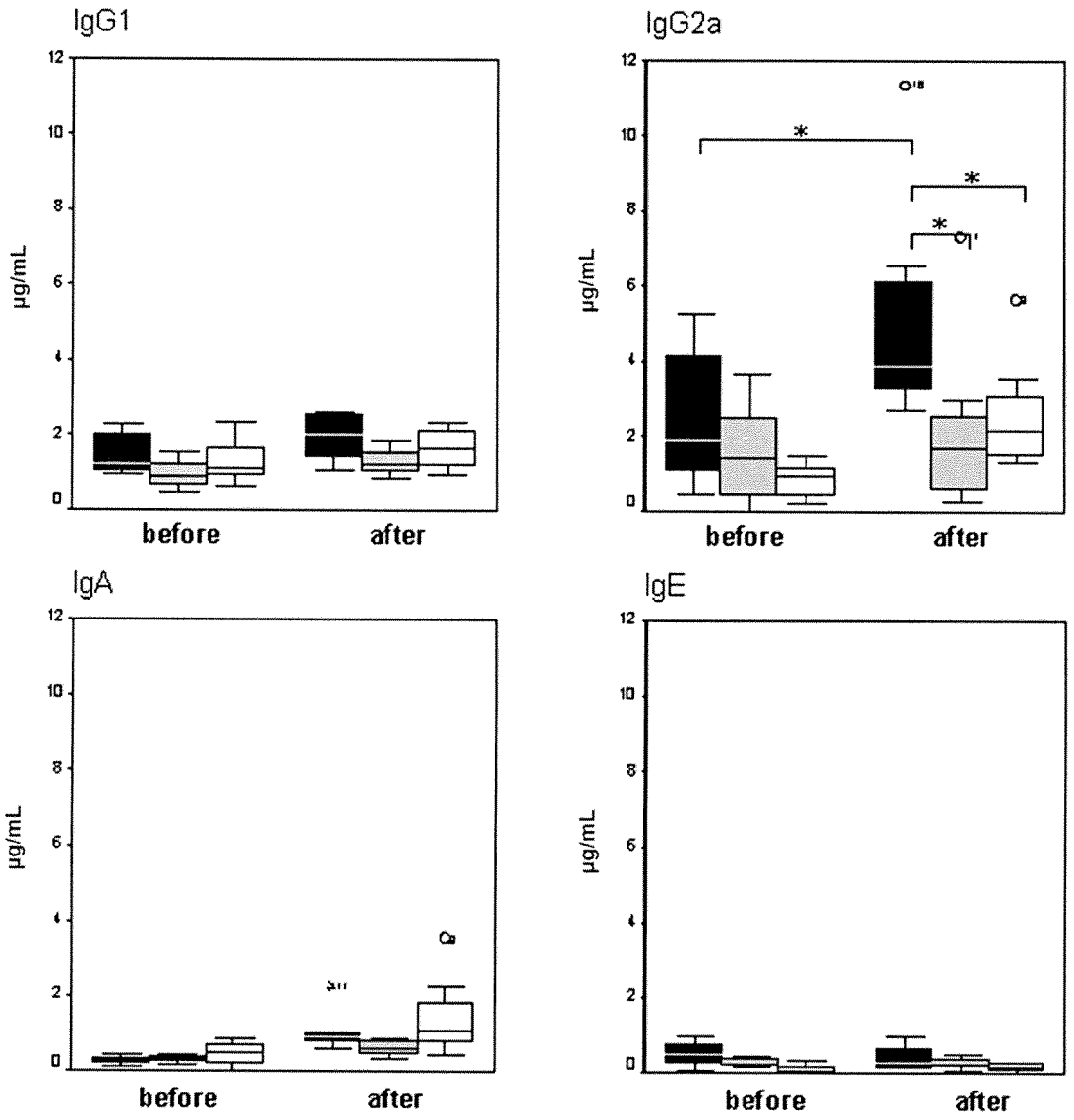

FIG 2. Birch pollen–sensitized mice (n = 8 per group) were treated orally with BP-loaded microparticles functionalized with AAL *(black columns)* or WGA *(gray columns)* or without functionalization *(white columns)*. Sera before and after treatment were analyzed for BP-specific IgG1 titer, IgE, and IgA titers (µg/mL serum) in ELISA. $*P < .05$.

Oral immunization of BALB/c mice

Mice were sensitized intraperitoneally with 10 µg birch pollen proteins in context with $Al(OH)_3$ in saline on day 0 and 23. As a therapeutic approach, oral feeding was performed 5 times each on 3 subsequent days (first, days 36-38; second, days 49-51; third, days 63-65; fourth, days 117-119; fifth, days 146-148). All groups (n = 8) received 200 µg BP encapsulated in microspheres functionalized with AAL or WGA or without lectin functionalization per gavage. The control group (n = 8) received nonencapsulated birch pollen proteins. Blood was taken from the tail vein on days −1 (preimmune serum), 22, 34, 48, 62, 113, 145, 175, and 215. Mice were killed on days 217 and 218.

Measurement of specific antibodies in ELISA

Birch pollen–specific IgG1 and IgG2a antibodies were measured following a standard ELISA procedure.[12] Briefly, birch pollen proteins (1 µg/well) or serial dilutions of mouse IgG1, IgG2a, IgE, and IgA standards (Southern Biotechnology Associates, Birmingham, Ala) were coated, blocked, and incubated with diluted sera (1:100 for IgG1 and IgG2a; 1:20 for IgE). Detection was performed by using rat antimouse IgG1, IgG2a, IgE, and IgA (PharMingen, San Diego, Calif), respectively, followed by incubation with peroxidase-conjugated mouse antirat IgG. As substrate, 2,2′-azino-bis(3-ethylbenzthiazoline)-6-sulphonic acid was used, and the reaction was detected at 405 to 490 nm. For IgE detection, tetramethylbenzidine (eBiosciences, San Diego, Calif) was used as substrate. The reaction was stopped with 1 mol/L sulfuric acid, and optical density was measured at 450 to 570 nm.

Proliferation assay

Spleens were removed under sterile conditions, minced, and passed through sterile nylon filters as described. After lyses of erythrocytes,

cells were washed and plated (100,000/well) into 96-well round-bottomed tissue culture plates (Nalge Nunc, Naperville, Ill). Cells were cultured with 2 μg/well birch pollen proteins, grass pollen proteins, 0.5 μg/well concanavalin A (Sigma), or medium alone for 4 days at 37°C. Culture supernatants were removed and the cultures pulsed with 0.5 μCi/well ^{3}H-thymidine (Amersham Biosciences, Uppsala, Sweden) for 16 hours. Cells were harvested, and ^{3}H-thymidine incorporation was measured in a liquid scintillation counter.

Cytokine assays

Culture supernatants of spleen cells stimulated in the proliferation assay were analyzed for IL-4, IL-10, and IFN-γ cytokine profile by using ready-to-use ELISA kits (eBiosciences) according to the manual instructions.

Data analysis and statistics

Data are presented as mean values ± SEMs. Statistical analysis was performed by using the Student *t* test to compare mean values (software SPSS version 11.5 for Windows SPSS GmbH Software, München, Germany). In each case, *P* values < .05 were considered statistically significant.

RESULTS

Lectin-functionalized microspheres bind to Caco2 cells

To exclude energy-dependent transport processes, binding studies of AAL or WGA-coated microspheres on Caco2 cells were performed at 4°C. As shown by immune fluorescence microscopy (Fig 1, *A*), relatively more fluorescein-cadaverine–labeled microparticles bound to the enterocytes when they were functionalized with lectins. In contrast, bare microparticles seemed to adhere randomly to the cells. The same could be demonstrated in a more quantitative manner by using Caco2 monolayers. WGA-coated and AAL-coated microparticles bound as much as 9 and 15 times better to the carcinoma cells than bare microparticles treated with glycine only (Fig 1, *B*).

Feedings of microparticles to sensitized BALB/c mice induce IgG2a when targeted to M cells

Antibody levels of individual mice of the differently treated groups were evaluated in ELISA (Fig 2). Sensitization of mice with birch pollen extract and aluminium hydroxide as adjuvant resulted in de novo induction of IgE and IgG1 antibodies in all mice, indicating successful induction of a T_H2-type immune response (data not shown). After 5 feedings, birch pollen–specific IgG2a levels were significantly higher ($P = .048$) in the group fed with AAL microparticles compared with the other groups of mice. In contrast, no alteration in the IgG1, IgE, and IgA levels could be observed.

Splenocytes of birch pollen–sensitized BALB/c mice proliferate specifically on antigen stimulation

Splenocytes from immunized mice responded specifically to stimulation with birch pollen extract. In the proliferation assay, birch pollen–stimulated splenocytes of mice fed with AAL microparticles were highest compared with the other groups. Stimulation was specific to birch pollen, because no response was obtained by incubation with grass pollen extract (Fig 3).

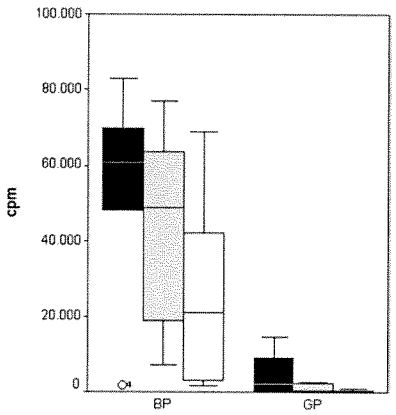

FIG 3. Mice (n = 8 per group) were sensitized to BP and afterward treated orally with BP-loaded microparticles. Splenocytes proliferated in all groups specifically to BP, but not with the control allergens of grass pollen *(GP)*. Feedings of these mice were performed with BP-loaded AAL microparticles *(dark gray columns)*, WGA microparticles *(gray columns)*, or uncoated microparticles *(white columns)*. *cpm*, Counts per minute.

Birch pollen–sensitized BALB/c mice produce IFN-γ when fed with AAL microparticles

Supernatants of birch pollen–stimulated splenocytes of the different mouse groups were investigated for IL-4, IL-10, and INF-γ cytokines. Both targeting strategies, using AAL for M cells and WGA for enterocytes, induced significantly elevated IL-10 and IL-4 levels. However, only AAL feedings induced significant elevated IFN-γ levels ($P = .0017$ to WGA microspheres; $P = .0007$ to bare, BP-loaded microspheres), as shown in Fig 4.

DISCUSSION

In this study, we provide evidence that immunomodulation toward T_H1 in an ongoing T_H2 response is possible by targeting M cells. When microparticles were targeted to α-L-fucose residues by functionalization with AAL, they induced birch pollen–specific INF-γ as well as IgG2a production. In contrast, no immunomodulation was observed with uncoated particles or WGA-coated microparticles targeting enterocytes.

Lectin-binding studies have revealed that in many species and at many organized MALT sites, the M-cell surface glycocalyx differs in carbohydrate composition from that of enterocytes. As a consequence, we looked for specific suitable agents that bind to M cells. In human beings, so far, no concrete difference could be identified.[17,23-25] From studies using mouse models, it is known that *Ulex europaeus* 1, a lectin specific for α-L-fucose, binds almost exclusively to the apical membrane of the

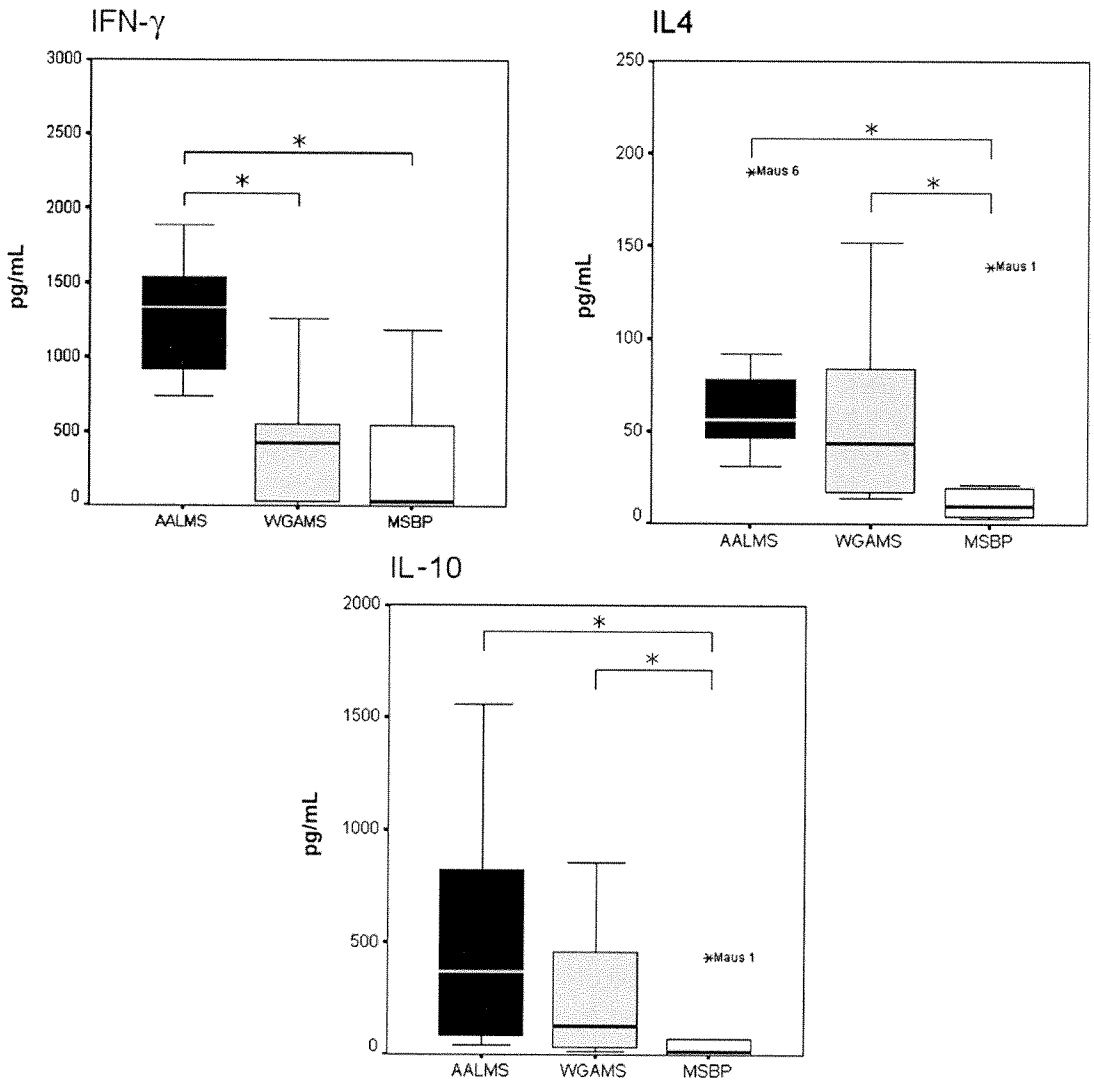

FIG 4. Supernatants of splenocytes of BP-sensitized mice (n = 8 per group), which were treated orally with BP-loaded microparticles, were analyzed for cytokine contents. WGA-coated and AAL-coated microparticles produced significantly more IL-10 and IL-4 than feedings with bare, BP-loaded microparticles *(MSBP)*. For IFN-γ, only AAL microspheres *(AALMS)* produced significant elevation of this cytokine. *WGAMS,* WGA microspheres. $*P < .05$.

Peyer patch M cell.[23,24,26,27] Because *U europaeus* 1 origin is a highly toxic plant, we aimed here to find an edible, nontoxic alternative showing similar binding properties. We chose AAL, a lectin capable of detecting α-linked fucosyl units regardless of the structure of the aglycone. It has been successfully used to examine fucoglycoproteins unreactive with the lectins from *Lotus tetragonolobus* and *U europaeus*.[28] Our working hypothesis was that AAL should be a promising candidate for targeting α-linked fucosyl residues expressed by M cells, at least of mice.

Recent crystallographic studies of AAL[7,8] have revealed its close structural similarity to the neuraminidase of *S typhimurium* LT2.[7] Crennell et al[29] also showed that the LT2 sialidase of *S typhimurium* has the same fold as the influenza virus neuraminidase, a well known virulence factor.[30] Both *S typhimurium* [31] and influenza virus[32] bind to M cells. It is therefore tempting to speculate that AAL also may bind to human M cells. With a pI>9 like the LT2 neuraminidase,[9] AAL has a positive charge[6]—a beneficial property, because surfaces with positive charges seems to be preferentially taken up by M cells.[33] Because *S typhimurium* selectively adheres to and invades murine and human M cells,[1] these features are already exploited in the design of attenuated vaccines to transport the antigen directly to the MALT for effective antigen uptake and strong T_H1 responses, even if one must admit that diarrhea is a common side effect.[34]

In previous studies, we generated PLGA nanospheres and could demonstrate that they effectively modulate an ongoing allergic response in BALB/c mice when applied

subcutaneously.[12] Besides the reduction of the T_H2 antibody IgG1 and increases in the allergen-specific T_H1 antibody IgG2a, the induction of INF-γ and IL-10 could be demonstrated, which both could contribute to the effectiveness of an immunotherapy against allergy.[35-38]

In the current study, mice allergic to birch pollen were fed with the different types of birch pollen protein–loaded microparticles, and their immunomodulating effect was investigated on the cellular and humoral level. Only when mice were fed with AAL-coated particles, they showed a significant increase of IgG2a synthesis. Because AAL and PLGA were LPS-free (data not shown), triggering of innate immune response was excluded. We suggest, therefore, that the M-cell targeting most likely led to IFN-γ production by allergen-specific T cells, which were responsible for this isotype switch. The IFN-γ and IgG2a formation was the most typical phenomenon by using the M-cell targeting strategy, whereas both M-cell and enterocyte targeting supported IL-4 as well as IL-10 production to a lower, although significant, extent.

Taken together, oral immunotherapy with AAL microparticles produced similarly strong allergen-specific immune response as treatment by the subcutaneous route.[12] The advantages offered by the presented delivery system are (1) protection of the vaccine during gastrointestinal transit by microencapsulation and (2) an effective delivery of antigen directed to the mucosal immune induction site (murine M cells) by surface-immobilized AAL, and therefore, a directed accumulation of the desired allergens to the intestinal immune system.

In a recent hyposensitization study of allergic patients, untargeted timothy grass pollen–loaded microparticles proved to reduce medication and symptom scores effectively when delivered orally.[39] Both T_H2 (implied by reduction in IL-5 mRNA) and antigen-induced proliferation of PBMCs were reduced in the treated patients compared with controls, whereas in our study, both T_H2 and T_H1 responses were increased in mice treated with allergen-loaded AAL microparticles. Comparison of these 2 studies is difficult because tolerance and immunity are not synonymous in both species.[40] Also, the size of the particles (~500 μm vs <10 μm in our study) and composition of the microparticles (methylacrylic acid copolymer vs PLGA) as well as time frame of application (10 weeks daily with increasing doses versus 5 times on 3 consecutive days) were different. In the hyposensitization study, the microparticles were too big for uptake, and the microcapsules dissolved immediately at a pH >6,[41] suggesting that released grass pollen allergens were taken up in soluble form by enterocytes and not by M cells. In contrast, in our study, most allergen-loaded microparticles were approximately 3 μm and therefore were suitable for cellular uptake. With additional functionalization of these microparticles, we were able to target murine Peyer patches, resulting in an active immunization on the humoral and cellular level. Moreover, in our experimental system, the allergens were released continuously.[18]

In the literature, low doses of oral antigen are described to lead to the activation of regulatory T cells,[42] which suppress immune responses by cognate interactions and IL-10 and TGF-β synthesis. Mowat et al[43] have shown that soluble antigens lead to systemic tolerance, whereas particulate antigens generally prime immune responses. In tolerance, it seems essential to pass intestinal epithelial cells and to escape capture by lamina-propria phagocytes.[41] Finally, ablation of Peyer patches does not affect tolerance.[44]

We demonstrate that antigen delivery to M cells can be mediated by AAL from the edible orange cup mushroom, which is able to target M cells *in vivo* and to support IFN-γ and IgG2a induction, at least in an animal model. Therefore, we conclude that AAL-functionalized microspheres may be used for successful oral administration of allergens. The similarity of AAL and *Salmonella* neuraminidase structures suggests that AAL could be a means for mucosal targeting in human patients, also.

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Histology and Histopathology

Lectin-binding sites in the epithelium of normal human appendix vermiformis and in acute appendicitis

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Summary. By using histochemical methods, the binding pattern of various lectins in the epithelium of normal human appendix vermiformis was assessed. In addition to plant and invertebrate sugar receptors with nominal monosaccharide specificity for α-L-Fuc (UEA-I), α-D-Man and α-D-Gluc (Con A), α-D-GalNAc (DBA), D-GalNAc (SBA, HPA) ß-D-Gal (RCA-I) and D-Gal (VAA), a mammalian ß-galactoside-specific lectin (MW, 14 kDa) was included in the applied panel. The apical surface of enterocytes presented binding sites for RCA-I on all cells, binding sites of UEA-I, DBA, SBA, HPA and VAA heterogeneously and no binding sites of Con A and 14 kDa. Binding sites of DBA, SBA, HPA, VAA and RCA-I within enterocytes were located primarily focally in a supranuclear position, whereas Con A and 14 kDa bound to the cytoplasm both in apical and basal cell parts.

In the follicle-associated epithelium more enterocytes expressed SBA- and VAA-binding sites than in the crypt epithelium. No differences between the lectin-binding pattern of M-cells and enterocytes were found in the follicle-associated epithelium. Intraepithelial macrophages were heterogeneously positive for the full panel of applied lectins. In contrast, intraepithelial lymphatic cells expressed binding sites only for RCA-I and less prominently for Con A, VAA and 14 kDa. Goblet cell mucus contained lectin-binding sites in a heterogeneous manner: binding sites for Con A were not detected in goblet cells for DBA, SBA, VAA and 14 kDa in less than 20%, for UEA-I in 20-40%, for HPA in 40-60% and for RCA-I in 60-100% of the goblet cells. Secreted mucus differed in its lectin-binding capacity from intracellular goblet cell mucus selectively by an increase of UEA-I, SBA- and RCA-I-binding sites and a lack of 14 kDa-binding sites. Comparative study of lectin binding to goblet cell mucin in another region of the large intestine, namely the rectosigmoid, demonstrated that DBA, SBA and 14 kDa bound mainly to the distal colon, while UEA-I and VAA labelling was selectively found in appendiceal goblet cell mucin.

Comparing the lectin-binding pattern in normal appendix epithelium and in appendicitis, the percentage of goblet cells expressing DBA- and SBA-binding sites in mucus globules was found to be about 4 times higher in appendicitis than in normal appendix. These results demonstrate that the expression of lectin-binding sites in appendiceal goblet mucin is specifically altered in appendicitis, indicating that there are selective changes of glycosylation of mucin in goblet cells mainly of the lower and middle crypt segment. Changes of lectin-binding pattern in appendicitis are discussed in connection with histochemical findings in inflammatory bowel disease.

Key words: Appendix, Appendicitis, Lectin

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Introduction

The human appendix vermiformis harbours a morphologically and functionally highly organized part of the gut-associated lymphoid system (Bockman, 1983; Spencer et al., 1985). The epithelium of the appendix vermiformis represents the receptor part of this appendiceal lymphatic organ (Owen and Jones, 1974). In this organ endogenous glycoconjugates are functionally involved in binding and uptake of intraluminal antigenic material and potentially pathogenic microorganisms (Neutra et al., 1987). Additionally, the epithelial lining of the appendix vermiformis establishes a protective coating against pathogenic noxes from the intestinal lumen (Sisson et al., 1971; Fischer et al., 1984). In particular, mucin glycoproteins secreted by the goblet cell population function as an important part of «non-specific» defence in addition to a complex array of immunological mechanisms (Filipe, 1979; Podolsky, 1989), thus warranting the thorough analysis of their glycocomposition and of any disease-associated alterations. Plant lectins and, recently, isolated mammalian lectins have already proved their value for histochemical detection of glycoconjugates in pathology (Gabius et al., 1991, 1993; Gabius and Gabius, 1993;

Danguy et al., 1994). Consequently, such a carbohydrate epitope-specific probe of mammalian origin will be employed in this stuy.

It is generally accepted that structural alterations in the secreted mucus are directly related to certain gastrointestinal diseases (Filipe and Branfoot, 1976; Filipe, 1979; Filipe and Fenger, 1979; Ehsanullah et al., 1982a,b, 1985; Reid et al., 1984). To addres the issue as to whether the expression of lectin-binding sites in human appendix vermiformis in appendicitis may change, we studied the epithelial lining of normal human appendix vermiformis with histochemical techniques using a panel of plant and mammalian lectins as tools. Comparative histochemical examination of appendicitis was performed to demonstrate inflammation-related changes of the glycoconjugate expression in the human appendix vermiformis.

Materials and methods

Tissue

21 appendix specimens were obtained at the time of surgery from patients operated on for suspected appendicitis. Normal mucosa samples of the sigmoid colon (n=6) and rectum (n=6) were obtained endoscopically from patients investigated for large bowel diseases, in whom no macroscopical or histological colonic lesions had been found. Specimens were fixed in 3.6% paraformaldehyde for paraplast-embedded sections. Histological diagnosis was confirmed by evaluation after conventional haematoxylin and eosin staining. Appendix specimens comprised 12 cases of normal appendix (control) and 9 cases of acute appendicitis.

Lectins

Biotinylated derivatives of concanavalin A (Con A), *Ulex europaeus* agglutinin-I (UEA-I), *Dolichos biflorus* agglutinin (DBA), soybean agglutinin (SBA), *Helix pomatia* agglutinin (HPA) and *Ricinus communis* agglutinin-I (RCA-I) were obtained from Sigma Co. (Deisenhofen, FRG). The ß-galactoside-specific lectin with a molecular weight of 14 kDa from bovine heart (14 kDa) and the galactoside-specific lectin from mistletoe (*Viscum album*, VAA), purified as described (Gabius, 1990), were biotinylated with biotinyl-N-hydroxysuccinimide ester in the presence of lactose to protect the active site (Bardosi et al., 1990; Gabius et al., 1992).

Histochemical processing

The sections were processed by rehydration, treatment with 1% hydrogen peroxide to block endogenous peroxidase activity, incubation with 0.1% periodate-treated BSA solutions to saturate unspecific protein-binding sites, incubation with 10 µg/ml biotinylated lectins (Con A, VAA, RCA-I, 14 kDa) or 40 µg/ml lectin (UEA-I, DBA, SPA, HPA), respectively, in phosphate-buffered saline, pH 7.4, containing 0.1% BSA for 4 h at room temperature and then for 1 h with ABC reagents, after through rinses. The formation of the coloured product, visualizing the probe-binding sites, was carried out by incubation for 30 minutes with the following solution: 15 mg 3-amino-9-ethylcarbazole dissolved in 3.75 ml dimethylformamide and added to 71.25 ml 0.1M sodium acetate buffer (pH 5.2), to which 0.75 ml 3% hydrogen peroxide solution was pipetted. Counterstaining was carried out with haematoxylin.

To ascertain the specificity of lectin-carbohydrate interaction, binding of the individual lectins was inhibited by co-incubation with a mixture of 0.2M sugar (inhibition of Con A by α-D-mannose; UEA-I by α-L-fucose; DBA, SBA and HPA by N-acetyl-D-galactosamine; VAA, RCA-I and 14 kDa by ß-lactose).

The extent of staining reactions was evaluated separately in enterocytes of the surface epithelium, in enteroyctes of crypts, in goblet cell secretory globules, in intraluminal mucus of the crypts, in intraepithelial macrophages (identified by the presence of intracytoplasmic inclusions) and in intraepithelial lymphocytes with respect to quantity of stained cells and intensity of staining. The quantity of stained cells (0=0%, (+)=0-20%, +=20-40%, ++=40-60%, +++=60-100%) and the staining intensity were grouped into categories, as given in detail in Table 1. Staining reactions in intracellular regions (apical cell surface, subapical region, supranuclear region, para-retronuclear region) of enterocytes were evaluated separately.

Results

Normal appendix vermiformis (see Table 1)

Histochemical reactions with Con A, UEA-I, DBA, SBA, HPA, VAA, RCA-I and 14 kDa in the epithelium of the human appendix vermiformis resulted in a characteristic staining pattern of epithelial and non-epithelium-derived cells (lymphocytes, macrophages) with respect to intensity of cellular staining, quantity of stained cells, and intracellular distribution of lectin-reactive sites as well as with respect to different cell types and intracellular and secreted mucus, respectively.

Con A (Fig. 1A)

Histochemical reactions with Con A in enterocytes of crypts and the surface epithelium resulted in a rather diffuse granular cytoplasmic staining, excluding the striated border. Intracellular and secreted mucus produced by goblet cells were free of Con A-binding sites. Staining of non-epithelial cells (lymphocytes and macrophages) was heterogeneous and weaker than in enterocytes.

UEA-I (Fig. 1B)

UEA-I bound intensely to the apical surface of most

Table 1. Binding of lectins to epithelium of normal appendix vermiformis.

LOCATION OF LECTIN BINDING	CON-A	UEA-I	DBA	SBA	HPA	VAA	RCA-I	14kDa
Surface enterocytes								
apical cell surface	-/0	+++/4	+/4	++/3	+++/2	+/3	+++/4	-/0
subapical cytoplasm	+++/4	+++/3	++/3	+++/3	+++/3	++/1	+++/4	+++/2
supranuclear cytoplasm	+++/4	+++/4	++/3	+++/3	+++/3	++/2	+++/4	+++/2
para-retronuclear cytoplasm	+++/4	++/3	(+)/2	-/0	(+)/3	-/0	(+)/1	+++/2
Crypt-enterocytes								
subapical cytoplasm	+++/4	+++/3	++/3	+/2	+++/3	(+)/2	+++/3	+++/2
supranuclear cytoplasm	+++/4	+++/3	++/3	+/2	+++/3	(+)/3	+++/3	+++/2
para-retronuclear cytoplasm	+++/4	++/3	(+)/1	-/0	(+)/3	-/0	(+)/1	+++/2
Goblet cells								
Intracellular mucus	-/0	+/2	(+)/3	(+)/3	++/3	(+)/2	+++/2	(+)/1
Luminal mucus	-/0	+++/3	(+)/3	+/3	++/3	(+)/2	+++/4	-/0
Intraepithelial macrophages	++/3	++/3	++/2	++/3	++/2	++/3	+++/3	+/1
intraepithelial lymphocytes	+/2	-/0	-/0	-/0	-/0	(+)/3	+++/3	(+)/1

The percentage of positive structures (cellular subsites, cells, mucus) is grouped into the categories: -, 0%; (+), 0-20%;+, 20-40%; ++, 40-60%; and +++, 60-100%. The intensity of staining reaction is grouped into the categories: 0, no staining; 1, weak, but significant staining; 2, medium staining; 3, *strong staining; and 4, very strong staining.*

enterocytes. In the cytoplasm of enterocytes a focal (patchlike) binding of UEA-I was observed in the subapical cell region and the Golgi region. The para-retronuclear region of enterocytes was either free of binding sites or rather diffusely stained. The Golgi *region of enteroyctes in the surface epithelium was* mostly more strongly positive than the Golgi region of enterocytes in crypts. Intracellular mucus was only moderately stained in a minority of goblet cells. Lectin-binding to mucus secreted by the goblet cells into the crypt lumen was more intense than to intracellular mucus. Intraepithelial lymphocytes were generally free of binding sites of UEA-I. Macrophages binding UEA-I *intensely, were found in the epithelium restricted to the* follicle-associated epithelium.

DBA (Figs 1C, 2B)

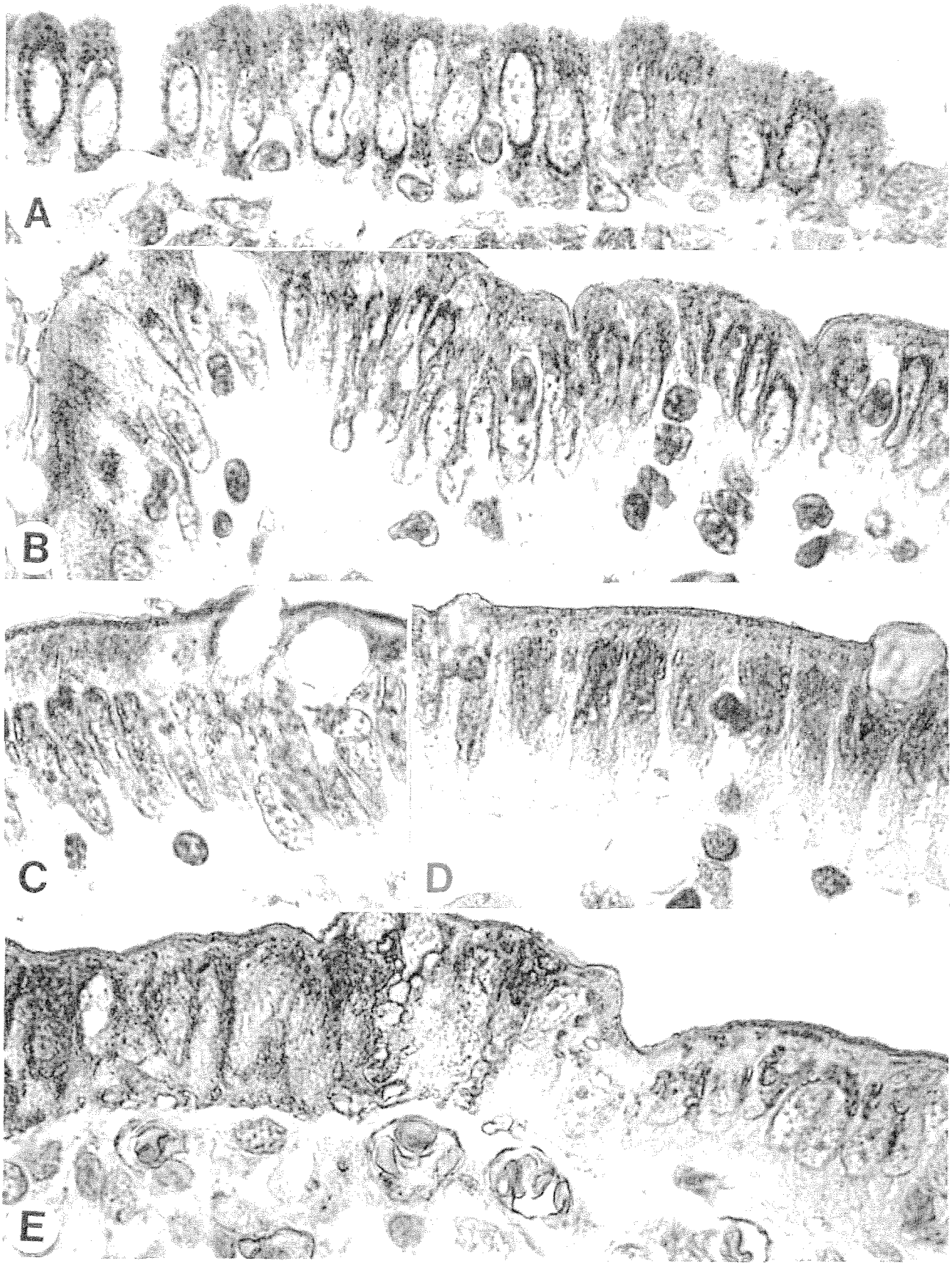
Binding of DBA to the apical surface of enterocytes was heterogeneous. In the cytoplasm of enterocytes an intense, partly vesicular, partly granular binding of DBA focally in about half of the cells was observed, whereas para-retronuclear staining was rather weak, granular and focal; overall restricted to about 16% of the cells. Para-retronuclear staining of enterocytes in crypts was even weaker than in the surface epithelium. Approximately 14% of the goblet cells contained secretory vacuoles with detectable binding sites for DBA. These goblet cells were restricted to the surface epithelium and the upper third of the crypts. Likewise, mucus with detectable DBA-binding sites in the crypt lumina was only observed in the upper third of the crypts. Intraepithelial lymphocytes were not labelled by DBA. Within intraepithelial macrophages DBA-binding sites were found focally associated with phagocytosed material.

SBA (Figs. 1D, 2A)

Binding of SBA to the apical surface of enterocytes was non-uniform, i.e. restricted to about half of the enterocytes. Enterocytes expressing SBA-binding sites at their apical surface usually also had respective binding sites in the cytoplasm. As noted for the surface, expression of binding sites in the cytoplasm of entero*cytes was heterogeneous.* Thus, only 70% of the enterocytes had respective binding sites, located selectively and focally within the subapical cytoplasm and the Golgi region, where vesicular dye precipitates were observed. Para-retronuclear SBA-binding sites were neither observed in the surface epithelium nor in the crypt epithelium. Staining reactions with SBA in the crypts were both weaker and restricted to fewer enterocytes compared to respective staining reactions in the surface epithelium. About 15% of the goblet cells contained secretory vacuoles with detectable binding sites for SBA. In the crypt lumina about a quarter of the mucus contained respective binding sites. Intraepithelial lymphocytes were not labelled with SBA. Within intraepithelial macrophages SBA-binding sites were associated with phagocytosed material.

HPA (Fig. 1E)

Binding of HPA to the apical surface of enterocytes and in their cytoplasm was heterogeneous, observed in more than 60% and 70% of the cells, respectively. The intracellular binding sites were located mostly selectively and focally within the subapical cytoplasm and the Golgi region, where fine vesicular dye precipitates were observed. In some appendix-cases epithelial cells of the crypts and the surface epithelium near the crypt openings exhibited a strong HPA-binding to the basolateral cell membrane and focally to the para-retronuclear cytoplasm. This cellular staining pattern did not occur in the follicle-associated epithelium. Thus, the border between follicle-associated epithelium and crypt-associated epithelium was clearly marked histochemically (Fig. 1E). About half of the goblet cells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contained secretory vacuoles with detectable HPA-binding sites. HPA-dependent staining was also noticed in about half of the secreted mucus in the crypt lumina (strong staining). Intraepithelial lymphocytes were free of HPA-binding sites, whereas macrophages of the follicle-associated epithelium demonstrated moderate staining reactions with HPA focally in granules of the cytoplasm.

VAA (Fig. 1F)

Binding of VAA to the apical surface of enterocytes was heterogeneous. In the cytoplasm of enterocytes a weak to moderate vesicular binding of VAA focally in about 50% of the enterocytes of the surface epithelium was observed. Staining intensity in the subapical cytoplasm was weaker than in the Golgi region. Para-

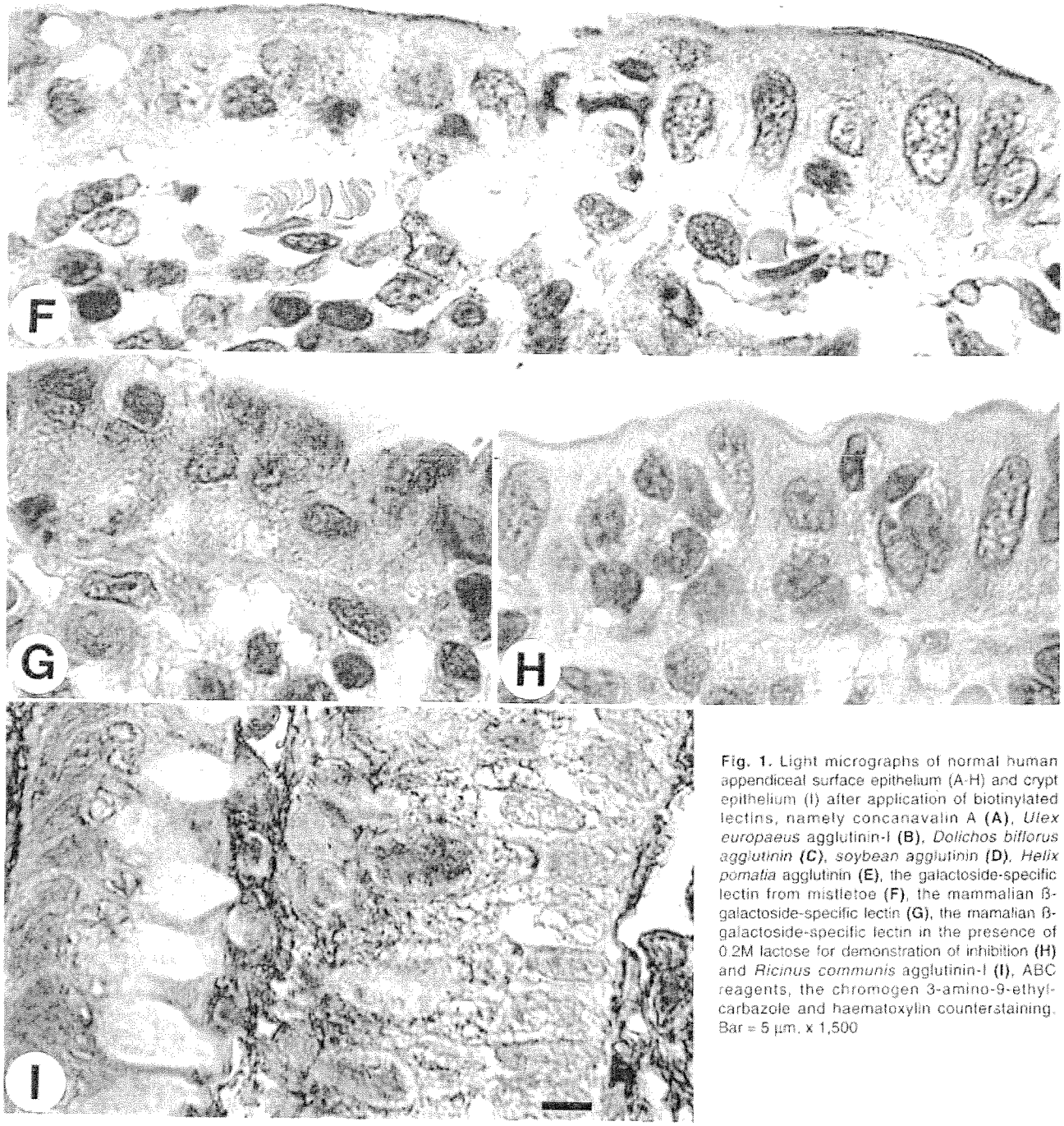

Fig. 1. Light micrographs of normal human appendiceal surface epithelium (A-H) and crypt epithelium (I) after application of biotinylated lectins, namely concanavalin A **(A)**, *Ulex europaeus* agglutinin-I **(B)**, *Dolichos biflorus agglutinin* **(C)**, *soybean agglutinin* **(D)**, *Helix pomatia* agglutinin **(E)**, the galactoside-specific lectin from mistletoe **(F)**, the mammalian ß-galactoside-specific lectin **(G)**, the mamalian ß-galactoside-specific lectin in the presence of 0.2M lactose for demonstration of inhibition **(H)** and *Ricinus communis* agglutinin-I **(I)**, ABC reagents, the chromogen 3-amino-9-ethyl-carbazole and haematoxylin counterstaining. Bar = 5 µm, x 1,500

retronuclear VAA-binding sites in enterocytes were not detectable under the given conditions. The same staining pattern was assessed in enterocytes of cyrpts. However, binding sites could only be detected in about 10% of the enterocytes. VAA-binding to secretory vacuoles could only be detected in few goblet cells. The staining intensity was moderate. Similarly, mucus secreted by goblet cells into the crypt lumina contained only focally-detectable VAA-binding sites. The lymphoid cell population of the epithelium was heterogeneous concerning the expression of VAA-binding sites. Macrophages in the follicle-associated epithelium clearly contained VAA-binding sites.

RCA-I (Fig. 1I)

RCA-I bound strongly to the apical surface of enterocytes. In the cytoplasm of enterocytes a focal granular binding of RCA-I was observed for the subapical and the Golgi region, whereas RCA-I binding sites in the para-retronuclear cell region were very rarely seen. Staining of enterocytes in the surface epithelium was stronger than in the crypt epithelium. About 90% of the goblet cells contained detectable RCA-I-binding sites within the secretory vacuoles. Comparison of RCA-I binding to intracellular goblet cell mucus and secreted mucus in the crypt lumina revealed a more intense histochemical reaction in the crypt lumina. Most of the lymphocytes (70%) and macrophages contained RCA-I-binding sites.

14kDa (Fig. 1G)

In contrast ot the other lectins, obtained from plants or invertebrates, the binding pattern of this mamalian ß-galactoside-binding protein serves as an indication for the presence of glycoligands with affinity to endogenous S-type lectins. Histochemical reactions with 14 kDa in enterocytes of crypts and the surface epithelium resulted in a moderate rather diffuse, in some cells focally intensified «patchy» staining, excluding the striated border. No clear difference concerning staining intensities, staining pattern and quantity of stained cells was observed between the surface epithelium and the crypt epithelium. 14 kDa was the only lectin within the applied panel that also labelled nuclei (in 10-20% of cells). 14 kDa labelled the basal membrane of the epithelium heterogeneously. Only a few single goblet cells contained detectable 14 kDa-binding sites within their secretory vacuoles. No 14 kDa-binding could be detected in mucus secreted into the cryptal lumen. 14 kDa-binding was seen in a few intraepithelial lymphocytes and some macrophages.

Acute appendicitis

Histochemical reactions with Con A, UEA-I, HPA and the three galactoside-binding lectins VAA, RCA-I and 14 kDa in the epithelium of acutely inflamed appendix vermiformis resulted in a characteristic staining pattern which was identical to the staining pattern in normal appendix vermiformis.

The binding pattern of DBA and SBA to appendix-epithelium, however, differed when sections of acute appendicitis and normal appendix were compared (Fig. 2A-D). Whereas DBA- and SBA-binding to enterocytes and non-epithelium-derived cells (lymphocytes, macrophages) was unchanged in acute inflammation, alterations of lectin binding to goblet cell mucus were found in acutely inflamed appendix.

Goblet cells exposing binding sites of DBA and SBA in their mucus droplets were more numerous in acute appendicitis (DBA: 54%, SBA: 59% of the goblet cells) than in normal appendix (DBA: 14%, SBA: 15% of the cells). Similarly, the quantity of DBA- and SBA-positive secreted mucus in the crypt lumina was higher in appendicitis than in normal appendix. These quantitative changes in appendicitis were apparently due to an increase of DBA- and SBA-reactivity of goblet cells in the lower and middle parts of the crypts.

Comparison of lectin binding in the normal human appendix vermiformis to lectin binding in the distal large intestine

Con A

In the distal large intestine Con A binding to enterocytes in crypts was weaker than that to surface enterocytes, whereas in the appendix Con A binding to crypt enterocytes was as strong as that to surface enterocytes.

UEA-I

In the distal large intestine UEA-I binding to crypt enterocytes was as strong as that to surface enterocytes, whereas in the appendix UEA-I binding to surface enterocytes was more intense than to crypt enterocytes. In the distal large intestine UEA-I binding sites were found neither in the goblet cell mucus nor in the secreted intraluminal mucus, whereas 20-40% of goblet cells in the appendix had UEA-I binding sites. Extracellular intraluminal mucus in the crypts of the appendix was stained even more strongly than in the goblet cells.

DBA

Whereas DBA binding to crypt enterocytes in the distal large intestine was weaker than to surface enterocytes, in the appendix binding of DBA in the supranuclear part of enterocytes was as strong in the surface epithelium as in the crypts. DBA binding to goblet cell mucus in the rectosigmoid was observed in 90% of the cells. In the appendix it was limited to approximately 14% of the goblet cells, located selectively in the surface epithelium and upper third of the crypts. In the rectosigmoid also, goblet cells free of DBA-binding secretory globules were detected in the crypt ground, suggesting that DBA-binding sites are an indicator of mucus production in «highly differentiated» cells.

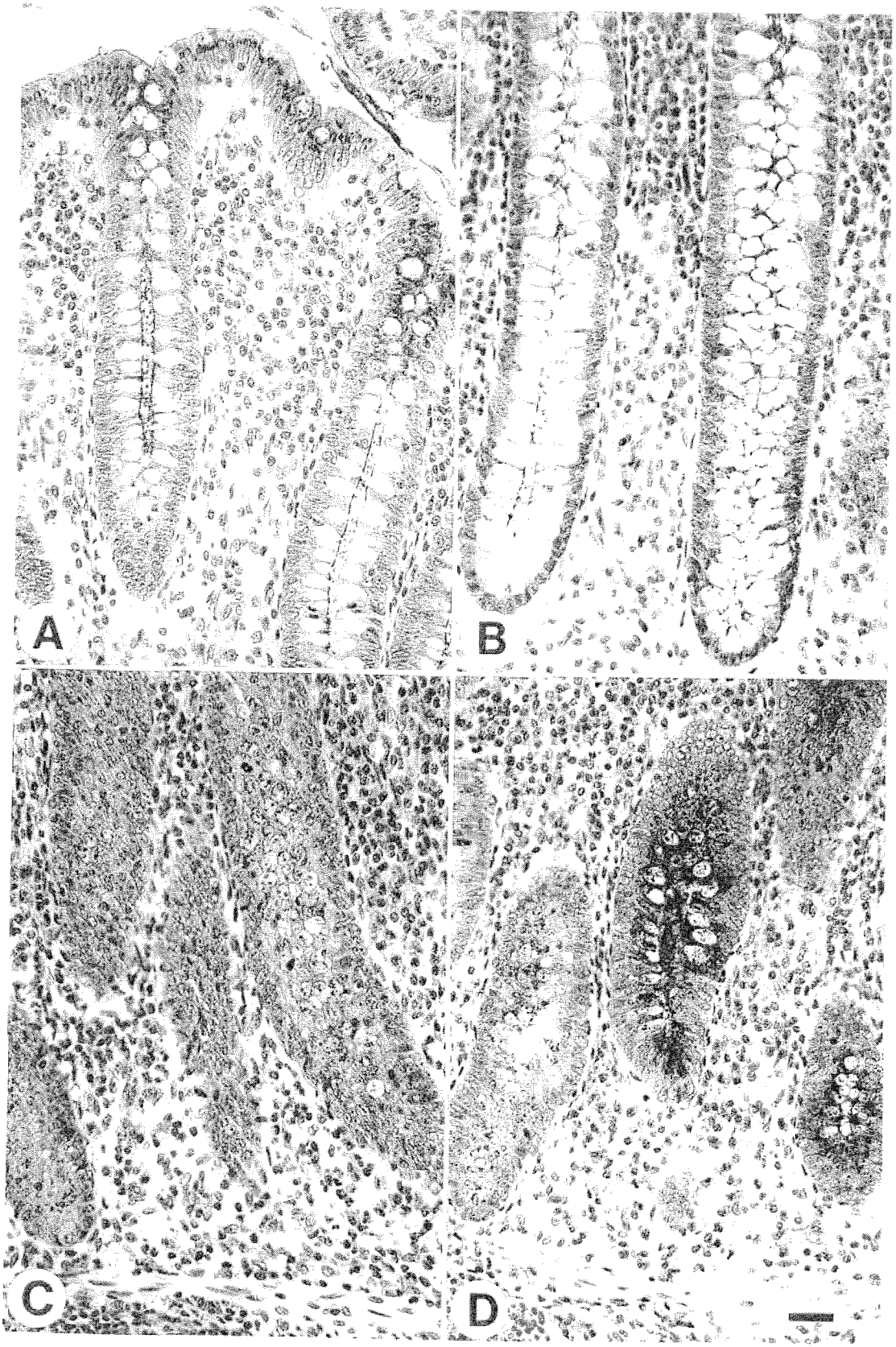

Fig. 2. Light micrographs of normal human appendiceal crypts (A,B) and appendiceal crypts in acute appendicitis (C, D) after application of biotinylated soybean agglutinin **(A, C)** and biotinylated *Dolichos biflorus* agglutinin **(B, D)**, ABC reagents, the chromogen 3-amino-9-ethylcarbazole and haematoxilin counterstaining. Bar= 24 µm. x 330

SBA

In the rectosigmoid, binding of SBA was equally weak in surface and crypt enterocytes. In contrast, in the appendix SBA binding was stronger in the surface enterocytes than in the crypts. Evident differences were noted between the number of SBA-binding goblet cells in the appendix and in the rectosigmoid; namely 80% compared to 15% of the cells were positive.

HPA

Binding of HPA to the epithelial lining of the rectosigmoid differed from the appendiceal binding pattern by a greater number of HPA-binding goblet cells (approximately 95% in the rectosigmoid versus 50% in the appendix).

VAA

VAA binding to enterocytes of the distal large bowel was weaker than in the appendix vermiformis. In the rectosigmoid, binding of VAA to the Golgi region was a weak as that to the subapical region of the enterocytes, whereas in the appendix binding to the Golgi region was pronounced compared to the subapical region. VAA-binding sites could neither be detected in the goblet cell mucus nor in the secreted mucus. In the appendix, the galactoside-binding mistletoe lectin bound both to intracellular and to secreted mucus.

RCA-I

In the distal large intestine no differences between the binding intensity of RCA-I in intracellular and in secreted mucus were detected, whereas in the appendix binding to secreted mucus was stronger than to intracellular mucus.

14 kDa

In the rectosigmoid, 14 kDa binding to surface enterocytes was more pronounced than to crypt enterocytes, whereas in the appendix vermiformis no differences were noted. The quantity of 14 kDa-binding goblet cells was much higher in the rectosigmoid than in the appendix vermiformis (10% versus 80%). In the rectosigmoid, 14 kDa-binding sites were detected in the secreted mucus, whereas this lectin failed to detect any suitably glycoligands in the appendix vermiformis.

Discussion

This study has analyzed the lectin-binding pattern in the epithelium of normal human appendix vermiformis. Comparative investigation of acute appendicitis revealed differences of lectin binding in mucus droplets of goblet cells and in secreted (luminal) mucus in the inflammatory state.

In the epithelium of normal human appendix vermiformis lectins differ in their affinity for different cellular components of enterocytes. SBA- and VAA-binding sites are restricted to the supranuclear and subapical cell regions. UEA-I-, DBA-, HPA- and RCA-I-binding sites are located both in the supranuclear and subapical cell regions and, quantitatively different, in the basal cell region. Binding sites of all these lectins (SBA, VAA, RCA-I, UEA-I, DBA, HPA) are focal in the cells, thus suggesting mainly binding to organelles supposedly of the Golgi apparatus and the smooth endoplasmic reticulum in the subapical cell region (Lee, 1987). Con A and 14 kDa bound to glycoligands in the cytoplasm of the supranuclear, subapical and basal cell regions. The preference of Con A for cytoplasmic staining can be attributed to the presence of oligomannose-like structures in the rough endoplasmic reticulum (Laurila et al., 1978; Fuhrman and Bereiter-Hahn, 1984).

Binding of lectins with similar specificity to a monosaccharide can exhibit dissimilarities. Taking galactoside-binding lectins like VAA and ricin as an example, the participation of structural features of the subterminal sugar moiety and anomeric linkage type significantly differ within this group (Lee et al., 1992, 1994). Both applied plant lectins (RCA-I, VAA) primarily recognize the terminal galactose residue with graded influence of the subterminal carbohydrate moiety and the anomeric linkage, whereas the mammalian lectin strongly binds to the Gal-ß-1,4-GlcNAc - disaccharide (Lee et al., 1992, 1994). Obviously, the similar nominal specificity to galactose will not guarantee a comparable extent of binding to cellular galactose-containing glycoconjugates, emphasizing the need to employ the tissue lectin for any functional correlations. It is interesting to note, with respect to the subcellular staining pattern, that from this group of galactose-binding lectins the mammalian lectin exhibited nuclear staining. In line with the biochemical data, RCA-I and VAA, exhibiting a comparatively low degree of selectivity to galactosides, stained macrophages and lymphocytes fairly well in contrast to the tissue lectin. As emphasized for galactoside-binding lectins, the GalNAc-specific lectins SBA, DBA and HPA similarly revealed notable differences in their binding pattern to certain cell types, namely enterocytes and goblet cells, reflecting measurable levels of dissimilarities in the fine structural ligand recognition.

Lectins differ distinctly in their affinity to the apical surface of enterocytes including M-cells, as indicated by different staining intensities, heterogeneity of binding to enterocytes or lack of apical surface binding. The apical surface of enterocytes is formed by microvilli with mucus closely attached to it (Forstner et al., 1973; Etzler, 1979). As lectin-binding sites at the apical surface of intestinal epithelial cells may be used as adhesion sites by bacteria, protozoa, or viruses (Gitler et al., 1985), lectin-binding sites in this location may play a role in intestinal infection (Sharon, 1987). Galactose residues, visualized in this study on the apical surface of M-cells and other enterocytes, could for example provide

suitable adhesion sites for *Entamoeba histolytica* with known suitable specificity of surface lectins (Petri et al., 1987) and preferential pathogenicity in the human appendix vermiformis. In this respect heterogeneity of enterocytes may be of interest. Having observed a correlation of presence of lectin-binding sites of DBA, SBA and VAA at the apical surface of enterocytes with the presence of respective binding sites in the supranuclear cell region, it can be assumed that the heterogeneity of respective lectin-binding sites at the apical surface may be due to differences of synthetic metabolism of enterocytes rather than due to accidental local variations of mucin-binding in the tissue specimen.

Remarkable quantitative differences exist for lectin binding between the enterocytes of crypts and the surface epithelium. Since enterocytes of the surface epithelium are derived from the crypt epithelium (Wolf and Bye, 1984) and build up a functionally specialized cover - the so called follicle-associated epithelium (Bockman, 1983) - it is reasonable to suggest that these differences may be differentiation-related. Apparently, these differences mainly affect the binding sites of SBA and VAA, which appear more frequently in enterocytes of the follicle-associated epithelium than in enterocytes of the crypt epithelium and of HPA which does not bind to the basolateral membranes of the follicle-associated epithelium.

Specialized cells of the follicle-associated epithelium, so-called M-cells, serve as antigen receptors of the gut-associated lymphatic system (Neutra et al., 1987). No clear-cut differences concerning the lectin-binding affinity of the apical cell surface of M-cells (in the vicinity of lymphocytes and macrophages) and non-M-cells (enterocytes not in contact with lymphocytes and macrophages) were found in our study. In these respects our findings are similar to previous results gained in histochemical studies on M-cells in the distal small intestine of mice and rats (Owen and Bhalla, 1983) and rabbits (Neutra et al., 1987). As M-cells elicit a mucosal immune response by transferring macro-molecules and bacteria to intraepithelial macrophages and lymphocytes (Owen, 1977; Wolf and Bye, 1984), we have studied the presence of glycosubstances in intraepithelial macrophages and lymphocytes which might be recognized by respective bacterial lectins in vivo. It is noteworthy that SBA binding to bone marrow-derived macrophages or their precursors differs markedly, eliciting differentiation-enhancing responses (Krugluger et al., 1994). Our staining reactions demonstrate that intraepithelial macrophages, lymphocytes and adjacent M-cells express a specific lectin-binding pattern with certain similarities and differences. As we have gathered preliminary information on variation of the lectin-binding pattern of macrophages in the gut-associated lymphoid tissue in relation to the location in different anatomical subsites of the appendix vermiformis, one can assume that differences of lectin-binding pattern might be related to environmental influences including exposure to phagocitable material.

Remarkable quantitative differences are assessed between the lectin-binding capacity of intracellular goblet cell mucus and extracellular secreted mucus in the crypt lumina. These differences may be due to mucin degradation by bacteria of the gut (Sato and Spicer, 1982). This study shows that goblet cell mucus in appendicits displays lectin-binding activities quantitatively different from goblet cell mucus in normal human appendix vermiformis. Similar changes of glycosylation of goblet cell mucus (increase of DBA and SBA binding, but not of HPA binding to goblet cell mucus) have been assessed in inflammatory bowel disease (Yoshioka et al., 1989). Thus, appendicitis can be regarded as an appropriate model system, demonstrating that changes of mucus glycosylation can be related to acute inflammation rather than a mixture of chronic and acute inflammatory processes as in inflammatory bowel disease. However, the qualitatively identical change of mucus glycosylation (in terms of lectin binding) as an inflammatory bowel disease is not necessarily related to malignant change, as has been controversely discussed in inflammatory bowel disease (Ahnen et al., 1987). The effects of mucosal inflammation on expression of appendiceal mucin lectin-binding sites has to our knowledge not previously been investigated. In our study in normal control appendices the percentage of goblet cells carrying DBA- or SBA-binding mucus is less than one third in all of 12 investigated specimens (ranging from less than 1% to 30%) and these goblet cells are restricted to the surface epithelium and to the upper third of the crypts. This appendiceal binding pattern of DBA is identical to the pattern in other subsites of the proximal large intestine, as reported in previous studies (Bresalier et al., 1985) and ascertained in the coecum (unpublished observation), but clearly differs from the distal large intestine, as observed by us and also reported previously (Bresalier et al., 1985). Although the precise functional implications of the observed alterations are at present not obvious, such changes clearly reflect regulation of glycosylation, warrating further studies.

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